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Involvement of cytochrome bc_1 complex and cytochrome c_2 in the electron-transfer pathway for NO reduction in a photodenitrifier, *Rhodobacter sphaeroides* f.s. *denitrificans*

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The electron-transfer pathway for NO reduction in a photodenitrifier, *Rhodobacter sphaeroides* f.s. *denitrificans*, was studied. A sample of membrane proteins containing cytochrome bc_1 complex and NO reductase activity was prepared from chromatophores using cholate/deoxycholate as detergents. The NO reductase activity was separated from the cytochrome bc_1 complex by ion-exchange chromatography in the presence of dodecyl maltoside. When duroquinol was used as an electron donor, NO was reduced in the bc_1-NO reductase preparation supplemented with cytochrome c_2. The reduction was inhibited by antimycin and myxothiazol. These results indicate that the cytochrome bc_1 complex, cytochrome c_2 and membranous NO reductase are involved in the electron-transfer pathway from quinol to NO in this photodenitrifier.

Cytochrome bc_1 complex; Cytochrome c_2; NO reduction; Denitrification; (*Rhodobacter sphaeroides*)

1. INTRODUCTION

Dentrification is an anaerobic bacterial respiration in which NO_3^- is reduced to the volatile products, N_2O and N_2, via the intermediates, NO_2^- and NO. It has previously been questioned as to whether NO is formed as a free intermediate in this process. Evidence has accumulated that NO is indeed a free intermediate [1-5] and that NO reductase is present in denitrifying cells grown anaerobically in the presence of NO_3^- [4,6,7]. It has been suggested that the NO reductase is tightly associated with the cell membrane [4,7-9], whereas dissimilatory NO_2^- reductase which produces NO is located in the periplasmic space [10,11]. Biochemical features of the NO reductase and the electron-transport system for NO reduction have not as yet been established.

A photodenitrifier, *Rhodobacter sphaeroides* fs. *denitrificans*, reduces NO_3^- to N_2 as an energy-transforming reaction other than O_2 respiration and photosynthesis [12]. The electron-transfer pathway for NO_2^- reduction in the photodenitrifier has been shown to involve cytochrome bc_1 complex and cytochrome c_2, by reconstituting the NO_2^--reducing system with a cholate/deoxycholate preparation of the cytochrome bc_1 complex, cytochrome c_2 and NO_2^- reductase [13]. Stoichiometric production of N_2O from NO_2^- in the reconstituted system occurred, and the bc_1 complex preparation itself possessed NO reductase activity [4].

Here, we report that the NO reductase activity can be separated from ubiquinol : cytochrome *c* oxidoredutase activity in a preparation of cytochrome bc_1 complex isolated by the method of Ljungdahl et al. [14]. The cytochrome bc_1 complex and cytochrome c_2 were involved in the electron-transfer pathway for NO reduction from quinol.

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2. MATERIALS AND METHODS

A green mutant strain of *R. sphaeroides* f.s. *ddenitrificans*

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IL106 was grown under denitrifying conditions in light as in [13]. Chromatophore membranes and cytochrome c_2 were prepared after disruption of cells using a French press as in [13].

Cytochrome bc_1 complex was isolated by two methods: one used cholate/deoxycholate as detergent and gel-filtration chromatography as reported in [15], where the resulting preparation had both ubiquinol:cytochrome *c* oxidoreductase and NO reductase activities; the other used dodecyl maltoside as detergent, and ion-exchange chromatography essentially as in [14], where ubiquinol:cytochrome *c* oxidoreductase and NO reductase activities were separated by the first step of DEAE-Biogel A chromatography (fig.1 and section 3).

NO reduction activity was assayed by determining N_2O produced from NO as in [4] using duroquinol or ascorbate-reduced PMS as the electron donors. Ubiquinol:cytochrome *c* oxidoreductase activity was assayed as in [16].

3. RESULTS AND DISCUSSION

NO reductase of this organism has previously been suggested to form a complex with cytochrome bc_1 complex from the result that the activity was found in a bc_1 complex preparation with seven major peptides obtained after solubilization of chromatophore membranes by cholate/deoxycholate and gel-filtration chromatography [4]. As shown in fig.1, however, NO reductase activity was separated from ubiquinol:cytochrome *c* oxidoreductase activity by DEAE-Biogel chromatography of membranes solubilized with dodecyl maltoside. Fractions with each activity were further purified by DEAE-Sepharose CL6B chromatography as described [14], followed by Sepharose 6B gel-filtration chromatography. Ubiquinol:cytochrome *c* oxidoreductase complex finally purified was composed of 4 polypeptides in SDS-polyacrylamide gel electrophoresis as described in [14]. The NO reductase preparation, so far as we obtained it, was composed of 7–8 polypeptides. It showed no ubiquinol:cytochrome *c* oxidoreductase activity. These results suggest that NO reductase is not tightly associated with the cytochrome bc_1 complex in the membrane.

Preliminary studies showed that the activity of NO reduction to N_2O with duroquinol as an electron donor in chromatophore membranes was decreased in the presence of cholate/deoxycholate (0.05% each) by about 70%. This was probably owing to dispersal of cytochrome c_2 from the vesicle interior; it was restored by addition of cytochrome c_2 externally (not shown). Activity was inhibited by 60% by antimycin and myxothiazol, which are inhibitors of ubiquinol:cytochrome *c* oxidoreductase. These results suggested that the NO reduction system in the photodenitrifier involves cytochrome c_2 and the cytochrome bc_1 complex. We studied this pathway further by reconstituting the NO-reducing system with a preparation of cytochrome bc_1 complex with NO reductase (cholate/deoxycholate preparation) and cytochrome c_2 (fig.2). The activity of NO reductase was found to be dependent on the concentration of cytochrome c_2. The apparent K_m for cytochrome c_2 was calculated via non-linear least-squares fitting to be 3.4 μM, with the V_{max} value being 0.092 nmol N_2O/s per nmol cytochrome c_1. Almost equal values of K_m and V_{max} were obtained when horse heart cytochrome *c* was used (not shown).

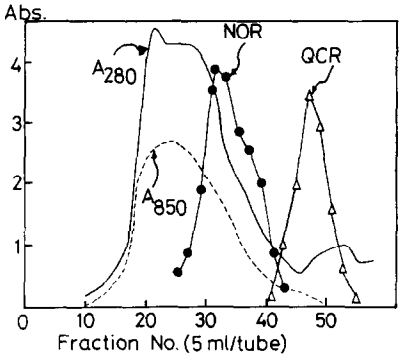

Fig.1. DEAE-Biogel A chromatography profiles of dodecyl maltoside extracts of chromatophores from *R. sphaeroides* f. sp. *denitrificans*. Chromatophore membranes (360 mg protein) from denitrifying-cultured cells were solubilized with dodecyl maltoside and applied to a column (2 × 16 cm) of DEAE-Biogel A as described in [14]. Absorbance at 280 nm; absorbance at 850 nm; (●—●) NO reductase activity; (Δ—Δ) ubiquinol:cytochrome *c* oxidoreductase activity.

Table 1 lists the effects of inhibitors of the cytochrome bc_1 complex on NO reduction and cytochrome c_2 reductase activity in the reconstituted system. The concentration of cytochrome c_2 used in the experiments was significantly lower than the optimal concentration for the activity of ubiquinol:cytochrome *c* oxidoreductase. This was because with higher concentrations of cytochrome c_2 the rate of NO reduction by duroquinol was determined mainly by NO reductase itself. In table 1, the NO reductase activity was inhibited about 50% by the inhibitors, indicating the involvement of the cytochrome bc_1 complex in electron transfer from quinol to NO. The cytochrome c_2 reductase

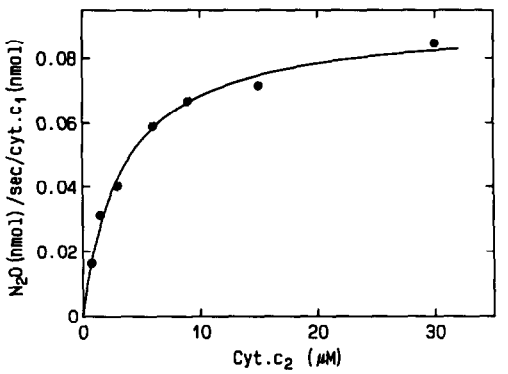

Fig.2. Dependence of NO reductase activity on concentration of cytochrome c_2. The reaction medium consisted of 50 mM Pipes-KOH (pH 7.0), the preparation of cytochrome bc_1 complex with NO reductase ([cytochrome c_1] = 0.67 μM) and various amounts of cytochrome c_2. Gas phase was 3% (v/v) NO in argon gas. The reaction was initiated by the addition of duroquinol (final concentration, 0.3 mM).

activity was inhibited about 90% under the same conditions. This difference in percentage of inhibition can also be explained by the notion that the rate for NO reduction can not only be determined by the cytochrome bc_1 complex but also by NO reductase in the absence of inhibitors. In the presence of inhibitors, on the other hand, the rates of NO reduction were similar to those of cytochrome c_2 reduction when compared on a per electron basis (for this purpose the values of NO reductase activity in table 1 must be doubled, since two electrons are required for the production of one molecule of N_2O). This indicates that the rate for NO reduction was mostly determined by electron transfer in the bc_1 complex under the conditions for inhibition. When ascorbate/PMS instead of duroquinol was used as the electron donor, no inhibitory effect of antimycin and myxothiazol was observed.

In this photodenitrifier, it has been shown that cytochrome c_2 mediates electrons from ubiquinol:cytochrome c oxidoreductase to photosynthetic reaction center complex, cytochrome c oxidase, and NO_2^- reductase. Reactions between cytochrome c_2 and these components are affected by electrostatic interaction. We have investigated whether this is the case for the reaction of NO reduction. Fig.3 shows NO reductase activity in the reconstituted system as a function of external ionic concentration. With increasing ionic concentration, the activity decreases. This confirms the function of cytochrome c_2 as a mobile electron carrier in electron transfer from duroquinol to NO in the reconstituted system.

These results led to the conclusion that cytochrome bc_1 complex and cytochrome c_2 take part in dissimilatory NO reduction in *R. sphaeroides* f.s. *denitrificans* as in the case of NO_2^- reduction [13,17]. (Although Kundu and Nicholas [18] have reported the failure of reduced cytochrome c_2 to act as an electron donor to purified NO_2^- reductase of this organism, we confirmed that NO_2^- reductase activity was dependent on the presence of cytochrome c_2 in a reconstituted system composed of purified bc_1 complex and NO_2^- reductase with duroquinol as the reductant.) In the

Table 1

Effect of inhibitors for the cytochrome bc_1 complex on NO reduction and cytochrome c_2 reductase activities in the reconstituted system

Inhibitors	NO reduction activity		Cyt c_2 reductase activity	
	nmol N_2O/s per nmol cyt c_1	Relative activity	nmol cyt c_2/s per nmol cyt c_1	Relative activity
None	0.053	100	0.495	100
20 μM Antimycin	0.027	50.9	0.074	14.9
20 μM Myxothiazol	0.026	49.1	0.059	11.8
20 μM Antimycin + 20 μM myxothiazol	0.022	41.5	0.039	7.9

The reaction medium consisted of 50 mM Pipes-KOH (pH 7.0), the preparation of cytochrome bc_1 complex with NO reductase ([cytochrome c_1] = 0.67 μM), 4.0 μM cytochrome c_2 and inhibitors. The gas phase and initiation of the reaction were similar to those in fig.2

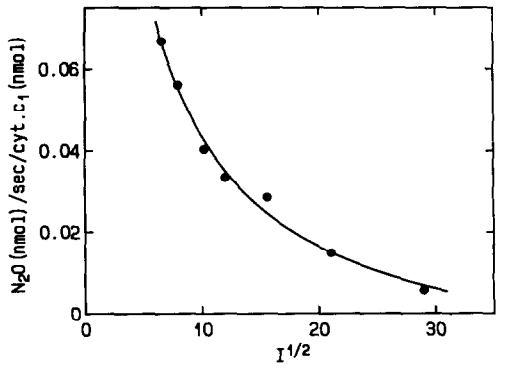

Fig.3. Dependence of NO reductase activity on ionic strength in the reconstituted system. The reaction medium consisted of 50 mM Pipes-KOH (pH 7.0), the preparation of cytochrome bc_1 complex with NO reductase ([cytochrome c_1] = 1.5 μM), 29.0 μM cytochrome c_2 and various amounts of KCl. Gas phase and the initiation of reaction were similar to those in fig.2.

reduction of NO_2^- to N_2O, NO is usually undetectable. Recently, it has been reported that NO can be trapped by extracellular hemoglobin [5], indicating that the NO produced is a freely diffusible intermediate. Thus, it is conceivable that NO produced in denitrification is caught and reduced by NO reductase at a higher rate than the diffusion of NO to the gas phase. The rapid trapping and reduction of NO might be associated with the sites of NO_2^- and NO reduction both of which are located on the periplasmic side of the cytoplasmic membrane [4]. Differently from other terminal reductases for denitrification, NO_3^-, NO_2^- and N_2O reductases, which are located in the periplasm of this bacterium, NO reductase was bound to the membrane and co-purified with the cytochrome bc_1 complex when cholate/deoxycholate was used. Until we tried the method of Ljungdahl et al. [14], we had failed to separate NO reductase from the cytochrome bc_1 complex by using several different methods. NO reductase may be associated with the cytochrome bc_1 complex in the membrane, and this association may play a role in the rapid trapping of NO produced by NO_2^- reductase located close to NO reductase. Thus, the cytochrome bc_1 complex, NO reductase, cytochrome c_2 and NO_2^- reductase may be closely associated when NO_2^- reduction occurs.

REFERENCES

[1] Payne, W.J. (1981) in: Denitrification. (Payne, W.J. ed.) pp. 54-67, J. Wiley, New York.
[2] Payne, W.J. and Grant, M.A. (1981) in: Genetic Engineering of Symbiotic Nitrogen Fixation and Conservation of Fixed Nitrogen. (Lyones, J.M., et al. eds) pp. 411-427, Plenum, New York.
[3] Zumft, W.G. and Frunzke, K. (1982) Biochim. Biophys. Acta 681, 459-468.
[4] Urata, K. and Satoh, T. (1985) Biochim. Biophys. Acta 841, 201-207.
[5] Goretshi, J. and Hollocher, T.C. (1988) J. Biol. Chem. 263, 2316-2323.
[6] Garber, E.A., Castignetti, D. and Hollocher, T.C. (1982) Biochim. Biophys. Res. Commun. 197, 1504-1507.
[7] Shapleigh, J.P. and Payne, W.J. (1985) J. Bacteriol. 163, 837-840.
[8] Grant, M.A., Cronin, S.E. and Hochstein, L.I. (1984) Arch. Microbiol. 140, 183-186.
[9] Shapleigh, J.P., Davies, K.J.P. and Payne, W.J. (1987) Biochim. Biophys. Acta 911, 334-340.
[10] Sawada, E. and Satoh, T. (1980) Plant Cell Physiol. 21, 205-210.
[11] Wood, P.M. (1978) FEBS Lett. 92, 214-218.
[12] Satoh, T., Hoshino, Y. and Kitamura, H. (1976) Arch. Microbiol. 108, 265-269.
[13] Urata, K. and Satoh, T. (1984) FEBS Lett. 172, 205-208.
[14] Ljungdahl, P.O., Pennoyer, J.D., Robertson, D.E. and Trumpower, B.L. (1987) Biochim. Biophys. Acta 891, 227-241.
[15] Takamiya, K., Doi, M. and Okimatsu, H. (1982) Plant Cell Physiol. 23, 987-997.
[16] Satoh, T. (1984) Arch. Microbiol. 139, 79-83.
[17] Sawada, E., Satoh, T. and Kitamura, H. (1978) Plant Cell Physiol. 19, 1339-1351.
[18] Kundu, B. and Nicholas, D.J.D. (1985) Arch. Microbiol 141, 57-62.